EXHIBIT 5

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Effects of Preexisting Immunity on the Response to Herpes Simplex-Based Oncolytic Viral Therapy

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ABSTRACT

Herpes simplex viruses (HSV) type 1 are the basis of a number of anticancer strategies that have proven efficacious in animal models. They are natural human pathogens and the majority of adults have anti-HSV immunity. The current study examined the effect of preexisting immunity on the response to herpes-based oncolytic viral treatment of hepatic metastatic cancer in a murine model designed to simulate a clinical approach likely to be utilized for nonneurological tumors. Specifically, the anticancer effects of NV1020 or G207, two multimutated HSV-1 oncolytic viruses, were tested in immunocompetent mice previously immunized with a wild-type herpes simplex type 1 virus. Mice were documented to have humoral as well as cell-mediated immunity to HSV-1. Tumor response to oncolytic therapy was not measurably abrogated by immunity to HSV at the doses tested. The influence of route of viral administration was also tested in models of regional hepatic arterial and intravenous therapy. Route of viral administration influenced efficacy, as virus delivered intravenously produced some detectable attenuation while hepatic arterial therapy remained unaffected. These results demonstrate that when given at appropriate doses and in reasonable proximity to tumor targets, HSV-based oncolytic therapy can still be expected to be effective treatment for patients with hepatic malignancies.

OVERVIEW SUMMARY

Herpes simplex viruses (HSV) are the basis of a number of anticancer strategies. The majority of humans have anti-HSV immunity. The current study examined the effect of preexisting immunity on the response to treatment of hepatic metastatic cancer using two different multimutated HSV-1-based oncolytic viruses. Route of viral administration influenced efficacy, as virus delivered intravenously produced some detectable attenuation while hepatic arterial therapy remained unaffected. These results demonstrate that when given at appropriate doses and in reasonable proximity to tumor targets, HSV-based oncolytic therapy can still be expected to be an effective treatment for cancer in patients with immunity to HSV.

INTRODUCTION

G207 IS A SECOND-GENERATION, multimutated, replication-competent herpes simplex virus type 1 (HSV-1). The virus is based on the wild-type HSV-1 F strain and contains deletions of both copies of $\gamma_1$34.5 (the gene that affects neurovirulence), and has an insertional inactivation of ICP6 (encoding the large subunit of ribonucleotide reductase) with the marker gene *lacZ* (Mineta *et al.*, 1995). NV1020 is another multimutated, replication-competent herpes simplex virus that is less attenuated than G207 and was originally designed for potential use as an antiherpes vaccine. The U_L56 gene has been deleted as has the HSV joint region, which includes one copy of the neurovirulence gene $\gamma_1$34.5 (Meigner *et al.*, 1988, 1990). These replication-competent mutant herpes viruses have shown promise in treating a multitude of solid tumors (Mineta *et al.*, 1995; Kucharczuk *et al.*, 1997; Toda *et al.*, 1998; Yoon *et al.*, 1998; Advani *et al.*, 1999; Carew *et al.*, 1999; Kooby *et al.*, 1999). The safety and utility of these agents are currently under investigation in various preclinical models to define their application in humans.

Preexisting immunity is a theoretical obstacle for virus-based therapies. Up to 90% of adults have circulating antibodies against herpes simplex virus (Corey and Spear, 1999). Most of these antibodies are generated against the viral glycoprotein

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coats, predominantly glycoproteins B, C, and D (gB, gC, and gD) (Glorioso *et al.*, 1984; Cantin *et al.*, 1987; Martin and Rouse, 1987; Nguyen *et al.*, 1992). These antibodies bind to viral particles and inhibit adhesion to and entry into target cells. They also function in inducing antibody dependent cell-mediated cytotoxicity (Glorioso *et al.*, 1984). Despite existing immunity wild-type HSV-1 is capable of evading the immune system, subsequently infecting and reinfecting the host (Davis-Poynter and Farrell, 1996). This may indicate that herpes-based viral therapies may be useful even in patients with preformed immunity to wild-type HSV.

Early investigation into the immune effect on herpes-based therapy has been promising (Herrlinger *et al.*, 1998; Chahlavi *et al.*, 1999). These studies, however, are limited by their use of direct intratumoral administration of virus. For nonneurological tumors, intravascular delivery is likely to be the method of administration utilized in the clinical setting. The current investigation was designed to determine if preexisting humoral and cellular immunity would inhibit the vascular delivery of virus-mediated antitumor treatment. Dose and route of administration were investigated to determine if either influenced the observed results. For those patients with no previous immunity to HSV, an immune response to the oncolytic viruses may occur during therapy, particularly if multiple doses are used. Therefore, studies were also performed to determine if the immune reaction generated in response to the oncolytic viruses differed from the response generated by the wild-type infection.

MATERIALS AND METHODS

Cell lines

CT-26 is a well-characterized murine colorectal carcinoma cell line that originated in BALB/c mice. This cell line was obtained from the National Cancer Institute (NCI) tumor repository (Fredericksburg, MD). P1HTR cells are murine lymphoma cells that express MHC-I and originated in BALB/c mice. Both cell lines were grown in RPMI supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 5 m*M* nonessential amino acids (GIBCO, Grand Island, NY). Vero cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS penicillin (100 U/ml), and streptomycin (100 μg/ml).

Virus

G207, originally a gift from S.D. Rabkin and R.L. Martuza (Georgetown Brain Tumor Center, Georgetown University Medical Center, Washington, D.C.), was constructed as described previously (Mineta *et al.*, 1995), with deletions of both copies of the $\gamma_1$34.5 gene and insertion of the *Escherichia coli lacZ* gene into the U_L39 sequence of the R3616 mutant.

NV1020 was clonally derived from R7020 and was originally obtained from B. Roizman (Pasteur Merieux Serums et Vaccins, Marcy l'Etoile, France) (Meigner *et al.*, 1988, 1990). It has a 15-kb deletion over the joint region of the HSV-1 genome. This deletion encompasses the region of the genome encoding ICP0, ICP4, latency associated transcripts (LATs), and the neurovirulence gene ($\gamma_1$34.5) and results in only one copy of these genes being present in the virus rather than two. It also has a 700-bp depletion of the endogenous thymidine kinase (TK) locus that prevents expression of the overlapping transcripts belonging to the U_L24 gene. A fragment of HSV-2 DNA from the *Hin*dIIIL region encoding several glycoprotein genes was inserted into the deleted joint region. An exogenous copy of the HSV-1 TK gene was inserted under the control of the α4 promoter. KOS was the wild-type strain used in these experiments.

Virus stocks were produced in WHO Vero cells seeded in roller bottles and infected at a multiplicity of infection (MOI) of 0.01. G207 and NV1020 were purified by size-exclusion chromatography (S500; Amersham Pharmacia Biotech, Piscataway, NJ) and concentrated by ultrafiltration (0.05-μm pore size, polysulfone hollow-fiber membrane; Spectrum, Laguna Hills, CA). KOS was purified by pelleting through a sucrose cushion (30% [w/v] in deionized water phosphate-buffered saline [D-PBS]). All virus preparations were formulated in D-PBS–10% glycerin and stored at −80°C.

Animals

All animal work was performed with the approval of the Memorial Sloan-Kettering (New York, NY) Institutional Animal Care and Use Committee. Male BALB/c mice were purchased from Taconic Farms (Germantown, NY). Animals were subjected to a 12:12 hr light:dark cycle, housed five per cage, and allowed food and water *ad libitum*.

Immunization

Animals were immunized by a standardized protocol. A total of 1×10^6 plaque-forming units (PFU) was administered in 500 μl of PBS via intraperitoneal injection. Control animals received 500 μl of PBS without virus via intraperitoneal injection. Ten days after inoculation, blood was obtained from test mice and the serum assayed for its neutralization potential as described below.

Assessment of humoral immunity

Humoral immunity was assessed by an *in vitro* viral neutralization assay. Briefly, 10 days after inoculation with either wild-type virus or PBS 1 ml of whole blood was harvested. Samples were left at room temperature for 1 hr and then centrifuged at 14,000 × *g* for 10 min. Serum was then harvested and stored overnight at 4°C. On the day of the blood harvest 1×10^6 Vero cells per well were plated into six-well plates (Costar, Corning, NY). These cells were allowed to settle overnight. Five microliters of serum was then harvested and mixed with 200 PFU of NV1020, G207, or KOS virus in a total of 100 μl of serum-free medium. The mixture was incubated for 1 hr at 37°C and then 700 μl of serum-free medium was added to each aliquot, bringing the total volume to 800 μl. The culture medium from each well of Vero cells was aspirated and the viral–serum aliquots were added to individual wells. All samples were processed in triplicate. Plates were incubated for 90 min with gentle agitation of the plates every 15 min. At the completion of incubation, wells were washed twice with serum-free medium and 1% agarose gel (Fisher Biotech, Fairlawn, NJ) was added to each well. Forty-eight hours later 0.0033% neu-

tral red solution (Sigma, St. Louis, MO) was added to each well. Plaques were counted 24 hr later.

Assessment of cellular immunity

Herpes-specific cellular immunity was characterized and assessed by a standard ^{51}Cr release assay. Briefly, effector cells were obtained from spleens of mice either immunized (test group) or not immunized (controls) against HSV. These cells were cocultured for 1 week after harvest with naive splenocytes that had previously been incubated with a fragment of the glycoprotein B protein identified as the immunodominant H-2K^b-restricted epitope for the HSV virus. This epitope is an octamer characterized as the sequence SSIEFARL (Dyall *et al.*, 1999). P1HTR cells labeled with this epitope or incubated with heat-inactivated virus were used as targets.

^{51}Cr-labeled target cells (1×10^4) were incubated at varying effector-to-target cell ratios for 4 hr. The supernatants were then harvested and assayed for radioactivity in an LKB-Wallace (Gaithersburg, MD) CliniGamma 1272 scintillation counter. The spontaneous release of the isotope was determined and maximum release was measured by adding 1% Triton X (Sigma) to each well. The percentage of specific cytolysis was calculated by the equation

$$(^{51}Cr \text{ experimental release} - {}^{51}Cr \text{ spontaneous release}/{}^{51}Cr \text{ maximum release} - {}^{51}Cr \text{ spontaneous release}) \times 100$$

Results were graphed and statistical analysis performed with a Student *t* test.

Model of hepatic metastases

A model of hepatic metastases as described elsewhere was utilized to assess the effect of existing immunity on viral efficacy (Lafreniere and Rosenberg, 1986). Briefly, animals were anesthetized and a small left subcostal incision was made, exposing the lower pole of the spleen. Tumor cells (5×10^4) were injected via a subcapsular injection in 300 μl of serum-free medium, using a 26-gauge needle. This approach delivers the injection into the portal venous system, generating an intrahepatic metastatic distribution.

Viral therapy

Twenty-four hours after intrahepatic tumor inoculation, the wound was reopened and, using a technique similar to that described, either 1×10^7 PFU ($n = 9$) or 1×10^6 PFU ($n = 5$) of the appropriate virus was administered in 300 μl of serum-free medium. Control animals ($n = 9$) received 300 μl of serum-free medium. In the initial experiments a second group of control animals ($n = 9$) was immunized with wild-type virus (KOS strain) and treated with serum-free medium to ensure that immunity alone did not confer any protection. For the intravenous model, therapy was given by administering 300 μl of serum-free medium and either 1×10^7 ($n = 5$) or 1×10^6 ($n = 5$) PFU of virus. Tail vein injection was used. Another group of control ($n = 10$) animals specific for this arm of the experiment was again given serum-free medium without virus. Animals were allowed food and water *ad libitum* for 2 weeks and were observed and weighed three times per week. At the conclusion of this period, the animals were killed, their livers were harvested, and the nodules were counted.

In a separate experiment, the animals were treated later in the tumor inoculation time course, either 3 or 7 days after injection of tumor, to assess if preexisting viral immunity might have a more pronounced effect on therapeutic efficacy at later time points. Mice were immunized as previously described by receiving wild-type virus (KOS strain) via intraperitoneal injection 14 days before injection of tumor. Mice were then separated into five groups ($n = 5$ per group), with all animals receiving 5×10^4 CT-26 cells via intrasplenic injection: (1) control animals receiving 300 μl of serum-free medium; (2) immunized mice treated on day 3; (3) immunized mice treated on day 7; (4) nonimmunized mice treated on day 3; and (5) nonimmunized mice treated on day 7. All mice were treated with 1×10^6 PFU ($n = 5$) of NV1020 in 300 μl of PBS via intrasplenic injection. Animals were allowed food and water *ad libitum* for 2 weeks and were observed and weighed three times per week. At the conclusion of this period, the animals were killed, their livers were harvested, and the nodules were counted.

Preimmunization with mutant viruses

Animals were immunized via intraperitoneal injection with 1×10^6 PFU of G207 ($n = 5$), NV1020 ($n = 5$), or PBS ($n = 10$). One week later, animals received a second intraperitoneal injection of the identical dose. After immunization with either G207 or NV1020, antibody inhibition of viral plaque formation was tested against all three strains used in this study. The neutralization assay was performed 10 days after the second injection. Animals required two doses of either G207 or NV1020 to generate titers high enough to reduce plaque formation by 10-fold in the neutralization assay. Mutated viruses are known to produce lower titers than wild-type virus and so this requirement was expected (Johnson *et al.*, 1986; Nguyen *et al.*, 1992; Morrison and Knipe, 1994). After confirmation of existing immunity, animals were inoculated with tumor and 24 hr later were treated with 1×10^6 PFU of virus via portal venous administration. Controls received serum-free medium. Two weeks later, animals were killed and liver nodules were counted.

Statistics

All statistical analyses were performed with a two-tailed Student *t* test unless otherwise specified.

RESULTS AND DISCUSSION

HSV-1-based oncolytic viruses have shown promise in preclinical studies against a multitude of solid tumors (Mineta *et al.*, 1995; Toda *et al.*, 1998; Yoon *et al.*, 1998; Advani *et al.*, 1999; Carew *et al.*, 1999; Kooby *et al.*, 1999; Bennett *et al.*, 2000). Some of these agents have begun phase I clinical trials and others are about to undergo such evaluations. Wild-type herpes simplex virus is a natural human pathogen and up to 90% of adults have circulating anti HSV-1 antibodies (Corey and Spear, 1999). Because of their neutralizing potential these antibodies may attenuate the efficacy of HSV-1-based oncolytic agents. In addition, existing cellular immunity may rapidly erad-

icate viral mutants designed to target cancer. The efficacy of adenovirus, another viral agent, is significantly attenuated by preexisting immunity (Yang *et al.*, 1994, 1995; Bouvet *et al.*, 1998). It is therefore important to characterize what effect preexisting immunity might have on herpes simplex-based viral therapy.

The present data regarding the interaction between the immune system and herpes-based therapy build on data from two previous investigations. In one study, Chahlavi *et al.* demonstrated that prior exposure to herpes simplex does not affect treatment with oncolytic viruses directly injected into tumors implanted into flanks of animals (Chahlavi *et al.*, 1999). Herrlinger *et al.* demonstrated an abrogated but not altogether abolished response to gene transfer using replication-incompetent HSV-1 vectors after direct injection into experimental intracranial tumors (Herrlinger *et al.*, 1998). These studies both utilize direct intratumoral inoculation as the method of therapy. While the information gained from these investigations is valuable, the results are not surprising since it has been shown that antibodies are unable to prevent cell-to-cell spread of virus, the likely mode of spread after direct tumoral injection (Simmons and Nash, 1985). This method of delivery decreases the likelihood that antibodies would have the opportunity to bind surface glycoproteins and inhibit viral adhesion and entry (Glorioso *et al.*, 1984).

The current study examines intravascular administration, a route more likely to be clinically useful. The results demonstrate that intraportal and intravenous viral injection may result in significant antitumor efficacy, even in animals with cellular and humoral immunity to wide-type herpes virus. Specifically, the efficacy of local vascular viral delivery (portal venous route) was studied and compared with the efficacy of systemic vascu-

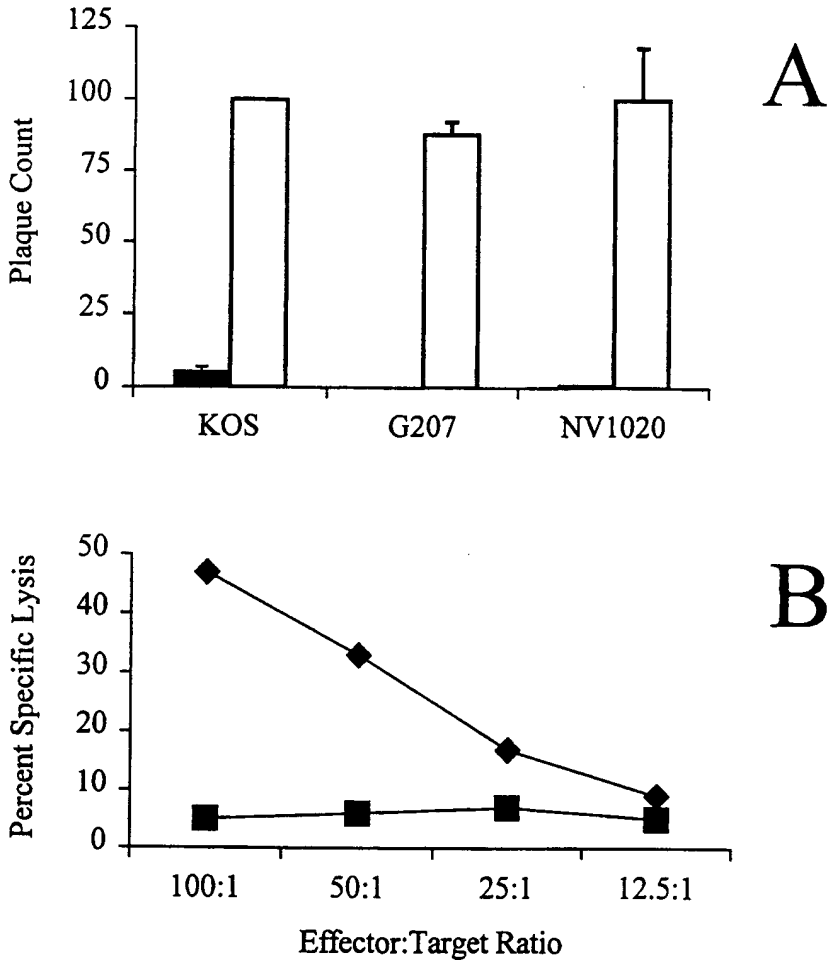

FIG. 1. (**A**) Representative neutralization assay. Animals were immunized with 1×10^6 PFU of wild-type virus and serum was harvested 10 days after inoculation. Five microliters of serum from immunized or nonimmunized mice was incubated with 200 PFU of NV1020, G207, or KOS in a total volume of 100 μl. Results are expressed as a percentage of the total plaques produced in the wells with virus and no mouse serum added. Solid columns represent plaques formed by virus incubated with serum from nonimmunized mice; open columns represent plaques formed by virus incubated with serum from immunized animals. All assays were performed in triplicate. (**B**) Representative CTL assay. Animals were immunized as described, spleens were harvested, and naive splenocytes labeled with a peptide fragment of the HSV-1 glycoprotein B molecule were used as antigen-presenting cells. P1HTR cells labeled with the same peptide fragment were used as targets. Diamonds represent percent specific lysis in immunized animals and squares represent nonimmunized animals.

lar delivery. Prior to the efficacy studies, humoral and cellular immunity were confirmed in this model, using neutralization (Fig. 1A) and chromium release assays (Fig. 1B). Neutralization assays were considered positive when there was a 10-fold decrease in the number of plaques formed. Results from these studies demonstrated that established antiviral immunity did not attenuate the antitumor efficacy at either of the doses tested when delivered locally. Viral therapy with G207 or NV1020 in immune and nonimmune animals reduced hepatic tumor nodule counts significantly compared with controls, with no difference between treatment groups after portal injection. High-dose portal viral therapy (1×10^7 PFU) with NV1020 reduced average tumor nodule counts (±SEM) from 270 ± 29 (controls) to 21 ± 0.6 in nonimmune animals and 4 ± 1.1 in immunized animals. After treatment with high-dose G207 the nodule counts were 3 ± 1.3 in nonimmunized mice and 13 ± 3.3 in immunized mice ($p < 0.001$) (Fig. 2). Low-dose portal therapy with 1×10^6 PFU of either G207 or NV1020 yielded results similar to those of high-dose therapy. Nodule counts for NV1020-treated immunized animals were 24.6 ± 10.2 for nonimmunized animals and 19.4 ± 8.4 for immunized animals, compared with control nodule counts of 88.4 ± 17. G207 reduced nodule counts to 21.6 ± 10.9 in nonimmunized animals and to 8.8 ± 5 in immunized animals ($p < 0.001$) (Fig. 2).

It may be that the amount of virus used, even at the lower dose, was sufficiently above the threshold of efficacy that viral neutralization did not manifest as measurable alterations in antitumor efficacy. With the current model, we could not move to a lower viral dose because of the unpredictable efficacy in nonimmunized animals. Utilizing an explanation similar to that proposed for direct tumoral injection, intraportal administration may have placed the virus sufficiently close to the tumor targets to avoid the immune system (Herrlinger *et al.*, 1998; Chahlavi *et al.*, 1999). Indeed, when the low dose of virus is administered intravenously, there was no longer a significant effect on tumor growth, suggesting that delivery of virus in proximity to tumor is an important factor in avoiding immune surveillance. Average nodule counts in mice with intravenously delivered NV1020 were as follows: 95 ± 21 (controls); 10.2 ± 4.7 (nonimmunized, high dose); 23 ± 9.1 (nonimmunized, low dose); 3.2 ± 1.5 (immunized, high dose); and 34.6 ± 12.3 (immunized, low dose). All groups significantly reduced tumor burden ($p < 0.05$) except the immunized low-dose group, which, as stated, yielded nonsignificant results (Fig. 3).

Studies of the effects of HSV immunity also have implications for the 10% of patients without established immunity to wild-type virus. Oncolytic viruses are likely to be administered in multiple doses, possibly generating an immune response specific to these mutants. Although data on immunization with wild-type HSV are highly suggestive that multiple administra-

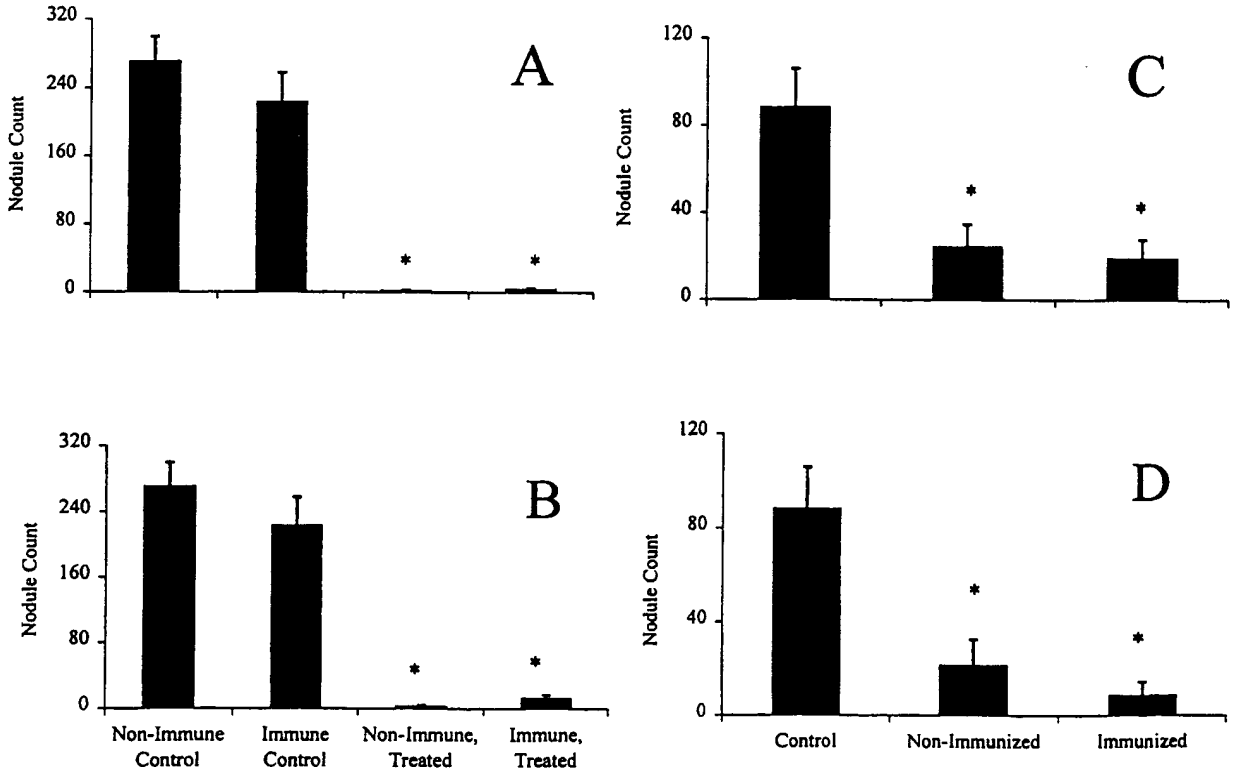

FIG. 2. Effect of immunization with wild-type KOS on subsequent intraportal therapy with 1×10^7 PFU of NV1020 (**A**) or G207 (**B**). Animals were inoculated with CT-26 and 24 hr later received therapy with virus. Controls received serum-free medium. Both immune and nonimmune controls were used in the initial study. In (**C**) and (**D**), intraportal therapy with 1×10^6 PFU of NV1020 (**C**) or G207 (**D**) was used. Immune controls were not used in this second experiment. Animals were killed 2 weeks after tumor inoculation and liver nodules were counted. Average nodule counts ± SEM per group are represented. *$p < 0.001$.

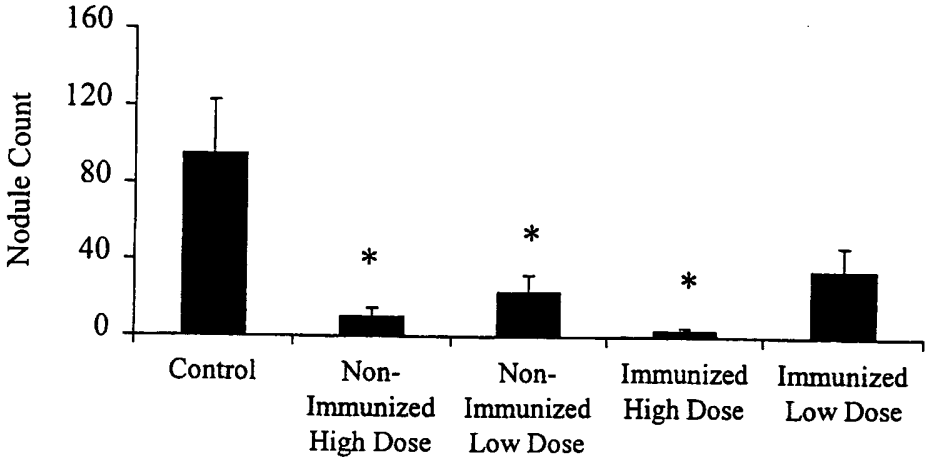

FIG. 3. Intravenous therapy with 1×10^7 or 1×10^6 PFU of NV1020. Animals were inoculated with tumor and 24 hr later received therapy with either a high or low dose via tail vein injection. Controls received serum-free medium via tail vein injection. Animals were killed 2 weeks later and liver nodules were counted. Average nodule counts ± SEM per group are represented. $*p = 0.05$.

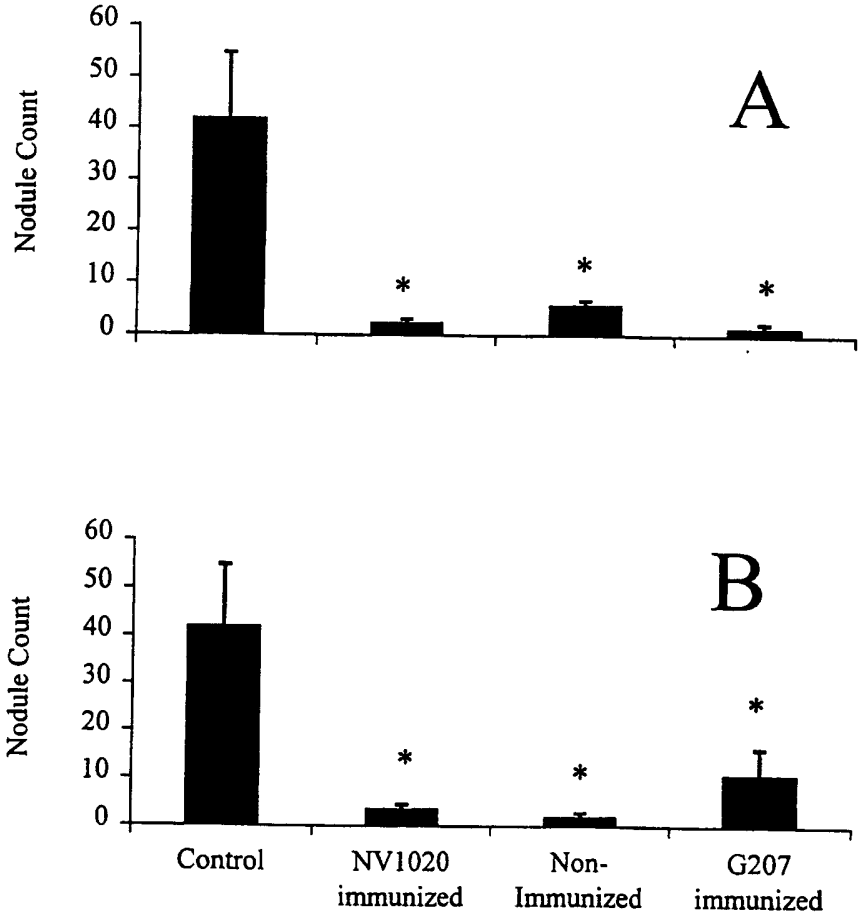

FIG. 4. Effect of preimmunization with G207 or NV1020 on subsequent treatment with these oncolytic viruses. Shown are liver nodule counts in animals preimmunized with G207 or NV1020 and treated with NV1020 (**A**) or G207 (**B**). Animals were immunized with two doses of either G207, NV1020, or PBS. After establishment of immunity, animals were injected with tumor into the liver and treated 24 hr later with 1×10^6 PFU of NV1020 or G207. Controls were treated with PBS. Columns and error bars represented average nodule count ± SEM. $*p < 0.03$.

tion of viruses may remain useful, we further examined if the development of specific immunity to the mutant oncolytic viruses might affect subsequent therapy. We also determined if this type of immunity would produce results different from those obtained after wild-type immunization. Although mutant viruses have been shown to stimulate both humoral and cellular immunity, they induce lower antibody titers than wild-type immunization (Nguyen *et al.*, 1992; Morrison and Knipe, 1994). In the current studies, a protocol utilizing two immunizations was required to generate antibody titers sufficient to produce neutralization *in vitro*. Despite immunity generated by the mutated viruses NV1020 and G207, as opposed to immunization with KOS wild-type virus, there was no attenuation of viral efficacy. Therapy with G207 or NV1020 was effective in animals preimmunized with either of the oncolytic viruses or not immunized at all. There were no differences between treated groups. NV1020 and G207 both reduced nodule counts from 45 ± 12 in control animals to 10 ± 0.8 nodules for each of the treated groups ($p < 0.03$) (Fig. 4). These results add to previously published data on improved efficacy of oncolytic therapy via multiple intratumoral viral injections in that vascular delivery was used. Furthermore, in these prior studies, the multiple injections of virus were given only days apart, in regimens unlikely to elicit vigorous antiviral immunity (Walker *et al.*, 1999).

To determine if immunity had an effect on abating the antitumor response of NV1020 given later in the tumor inoculation time course, animals were immunized with KOS wild-type virus, given tumor 14 days later, and treated either 3 or 7 days after tumor inoculation. The results from this experiment showed again that immunity did not abrogate antitumor efficacy of NV1020. The immune animals treated at 3 days had nodule counts that were significantly reduced from that of controls. Control animals had 152.5 ± 29 nodules, while those animals who were immunized and treated 3 days after tumor inoculation had 57.6 ± 8.1 nodules ($p < 0.01$). By day 7, treatment with virus, with or without prior immunization, did not significantly reduce tumor burden, likely because of the overwhelming tumor burden at this point.

At the doses tested, circulating antibodies have minimal measurable effects on viral oncolytic therapy. The explanation for this may lie in the fact that herpes simplex viruses have the ability to use a variety of mechanisms to target cells and therefore bypass existing immunity. Local regional therapy may allow HSV to evade the immune system purely by the proximity of the site of viral administration to tumor targets. Herpes simplex-based oncolytic viruses show promise as antineoplastic therapy. This article adds to a growing body of literature addressing essential preclinical considerations that encourage studies of these viruses in the clinical therapy of cancer.

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